

# The effect of *Echinacea purpurea* on the morphology, angiogenic activity and vascular endothelial growth factor (VEGF) concentration of murine L-1 sarcoma tumors

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## Abstract

**The aim** of the present study was to evaluate the effect of feeding mice *Echinacea purpurea*-containing drugs on 1) neovascular response induced in mice by L-1 sarcoma cells 3 days after intracutaneous grafting 2) morphology, angiogenic activity and VEGF concentration of murine sarcoma L-1 tumors 7 days after intracutaneous cells transplantation.

**Material and Methods:** *Echinacea purpurea*-containing drugs: Immunal forte tablets (LEK, Slovenia) and Echinapur tablets (Herbapol Poznań), 0.6 mg per day. Animals: inbred Balb/c mice 8-10 weeks old. Tumor cells: murine L-1 sarcoma from Warsaw Cancer Center Collection. Methods: cutaneous angiogenesis assay according to Sidky and Auerbach, VEGF concentration measurement (ELISA) and histological evaluation of L-1 sarcoma tumors 7 days after transplantation of cells.

**Results:** Echinapur diminished angiogenesis induced by murine L-1 sarcoma cells 3 days after intradermal cells injection. Both Echinapur and Immunal reduced VEGF concentration in L1 sarcoma tumor tissue, evaluated 7 days after intradermal cells grafting. There were no differences in mass and diameter of tumors between investigated and control groups. In *Echinacea*-fed groups, however, there were less small blood vessels at a margin of tumor.

**Key words:** *Echinacea purpurea*, L-1 sarcoma, mice.

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## Introduction

Use of *Echinacea purpurea* (purple coneflower), herb native to the North American prairies, has a long history. North American Indians used this plant internally and topically as antiseptic, anti-inflammatory, and analgesic drug. They used aerial and underground parts of the plant, in the form of infusion, succus or decoction, for curing burns, snake and insect bites, and as general immunoenhancers. Now, this plant is one of the most important world' medicinal herbs, widely used for the treatment of upper respiratory tract

infections [1]. We previously reported that *Echinacea purpurea* extracts enhance immunity in mice and stimulate various functions of human blood leukocytes *in vivo* [2-7]. Studies in cancer patients undergoing chemotherapy showed that polysaccharide fraction isolated from *Echinacea purpurea* may counteract the chemotherapy-induced leukopenia [8]. It was described that *Echinacea* extracts were able to induce apoptosis of human cancer cell lines by increasing caspase activity and promoting nuclear DNA fragmentation. It was also found that flavonoids and polyphenols, present in *Echinacea*, inhibit activities of metalloproteinases and some

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serine proteases important for angiogenesis, and inhibit VEGF receptor phosphorylation [9-12]. We recently reported, that *Echinacea purpurea* extract diminished neovascular reaction induced in mice skin by human cancer cells [7].

The aim of our present study was to evaluate the effect of feeding mice *Echinacea purpurea*-containing drugs on 1) neovascular response induced in mice by murine cancer cells 3 days after intracutaneous grafting and, 2) morphology, angiogenic activity and VEGF concentration of murine sarcoma L-1 tumors, 7 days after intracutaneous cells transplantation

## Material and Methods

*Echinacea purpurea*-containing drugs: Immunal forte tablets (LEK, Slovenia) and Echinapur tablets (Herbapol Poznań), 0.6 mg per day.

### Animals

Inbred Balb/c mice 8-10 weeks old. Mice have been of local laboratory breed, weighing 20 g each. Animals were handled according to the Polish law on protection of animals and NIH standards. All experiments were accepted by the local Ethical Committee.

### Tumor cells

Murine L-1 sarcoma cells from *in vitro* culture stock were delivered from Warsaw Oncology Center Collection and then passaged through three generations of Balb/c mice as previously described [13].

### Cutaneous angiogenesis assay

According to Sidky and Auerbach, with some modifications [13]. Briefly, multiple 0.05 ml samples of  $2 \times 10^6$  L-1 sarcoma tumor cells were injected intradermally into partly shaved, anesthetized Balb/c mice. In order to facilitate the localization of cell injection sites, the suspension was colored with 0.1% of trypan blue. Next, mice were fed *Echinacea* preparations in daily dose of 0.6 mg for 3-5 consecutive days. Some mice after 72 hours were sacrificed with lethal dose of Morbital. All newly formed blood vessels were identified and counted in dissection microscope, on the inner skin surface, at magnification of 6x, in 1/3 central area of microscopic field. Identification was based on the fact that new blood vessels, directed to the point of cells injection are thin and/or differ from the background vasculature in their tortuosity and divarications.

The other mice were fed *Echinacea* drugs for 5 days. At the day 7<sup>th</sup> mice were sacrificed with lethal dose of Morbital, the tumors were removed and weighted.

### Histological evaluation of L-1 sarcoma tumors 7 days after transplantation of cells

Half of excised tumors (6 lesions from each group) was fixed in 10% formalin solution. Next, the specimens were dehydrated in increased concentrations of alcohol and

embedded in paraffin. Paraffin tissue block was sectioned on 4 µm thin sections. The specimens were contrasted by hematoxyline and eosine for first screening light microscopic examination.

The second half of removed tumors (6 lesions from each group) was pooled within groups, and frozen at -78°C (suspended in PBS in proportion 100 mg per 1 ml) for later VEGF measuring.

### Measurement of VEGF concentration

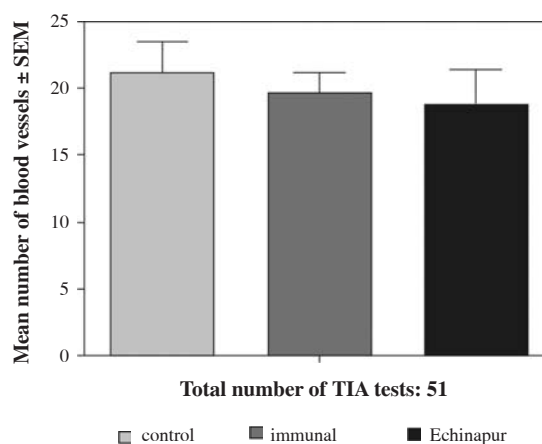
The tumour samples collected on the day seven after tumor cells grafting were homogenized with an ultrasonic disrupter VirSonic (Virtis) for 2 minutes, at frequency 22.5 KHz. Cytokine levels were determined using standard ELISA R&D kits for mouse VEGF, according to producer instructions. From each material 6 repetitions were established. Optical density was measured at 450 nm using spectrophotometric reader Elx800 (Biotek Instruments, Inc., USA). Cytokine concentration was expressed as pg/ml.

### Statistical evaluation of the results

One way analysis of variance ANOVA, Tukey's Multiple Comparison Test (GraphPad Prism software package).

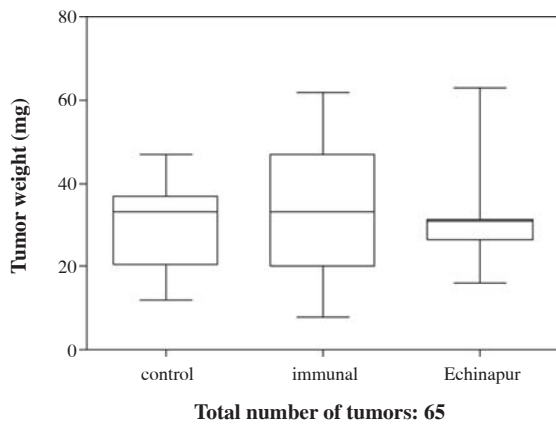
## Results

Echinapur diminished angiogenesis induced by murine L-1 sarcoma cells, as evaluated 3 days after intradermal cells



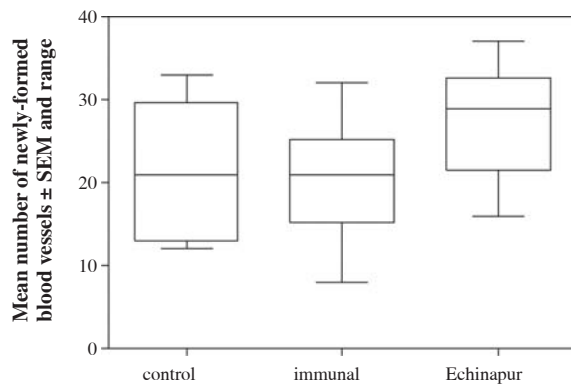
Tukey's Multiple Comparison Test	Mean diff.	q	Significant? p<0.05?	Summary
Control vs. Immunal	1.540	2.950	No	ns
Control vs. Echinapur	2.340	4.483	Yes	**
Immunal vs. Echinapur	0.8000	1.533	No	ns

**Fig. 1.** Neovascular reaction after grafting of L-1 sarcoma. Recipients of tumor cells were fed *Echinacea* for 3 days after grafting



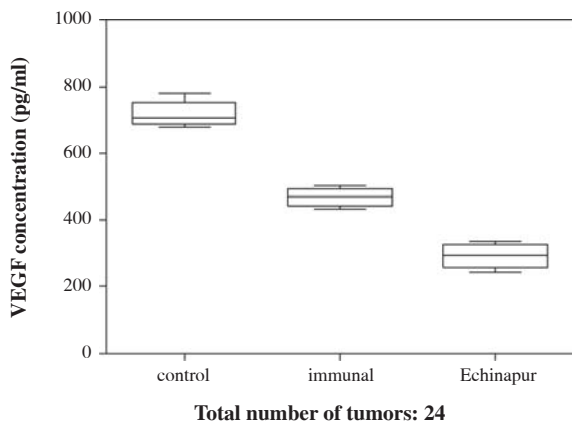
Tukey's Multiple Comparison Test	Mean diff.	q	Significant? p<0.05?
Control vs. Immunal	-2.750	1.130	No
Control vs. Echinapur	-0.7475	0.2797	No
Immunal vs. Echinapur	2.002	0.7493	No

**Fig. 2.** Mass of tumors collected from L-1 sarcoma grafted mice fed *Echinacea* for 5 days (mean ± SD and range)



Tukey's Multiple Comparison Test	Mean diff.	q	Significant? p<0.05?	Summary
Control vs. Immunal	1.833	0.8577	No	ns
Control vs. Echinapur	-5.300	2.364	No	ns
Immunal vs. Echinapur	-7.133	3.182	No	ns

**Fig. 3.** Neovascular reaction induced by tumor cells collected from mice fed *Echinacea* for 5 days. Total number of TIA tests: 34



Tukey's Multiple Comparison Test	Mean diff.	q	Significant? p<0.05?	Summary
Control vs. Immunal	249.6	21.27	Yes	***
Control vs. Echinapur	424.5	36.16	Yes	***
Immunal vs. Echinapur	174.9	14.90	Yes	***

**Fig. 4.** VEGF content in tumors collected from mice grafted with L-1 sarcoma cells and fed *Echinacea* for 5 days (mean ± SD and range)

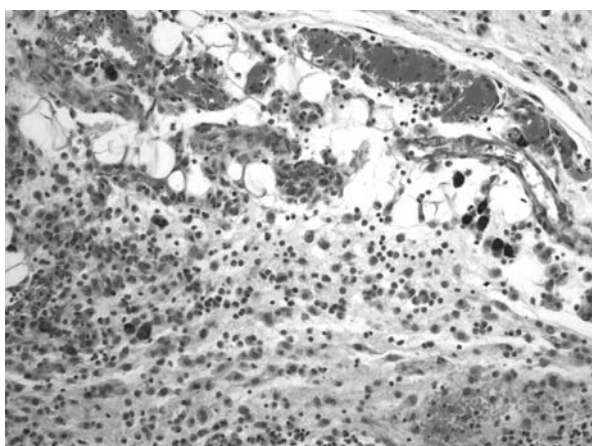
injection. Immunal did not change neovascular reaction (Fig. 1). Both drugs did not reduce tumor mass (Fig. 2) and tumor cells angiogenic activity (Fig. 3), but highly significantly diminished VEGF concentration (Fig. 4) in L1 sarcoma tumor tissue, evaluated 7 days after intradermal cells grafting.

Histologically, there were no changes in diameter of tumors between investigated and control groups. Also, there were no inflammatory infiltrations and areas of necrosis in all groups. In *Echinacea*-fed groups, however, there were less small blood vessels at a margin of tumors (Figs. 5 and 6).

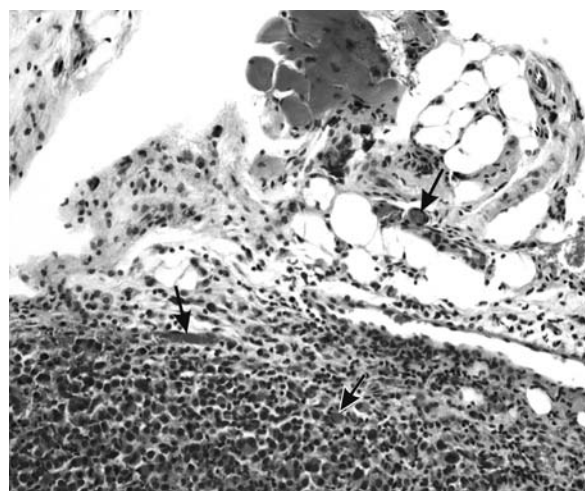
## Discussion

The results of the present study demonstrated that Echinapur, *Echinacea purpurea* derived drug, diminished neovascular reaction induced in mice skin by syngeneic L-1 sarcoma cells. This effect was not observed when mice were fed for 3 days with other *Echinacea purpurea* derived drug, Immunal. However, both drugs administered to tumor recipients for 5 days, reduced VEGF concentration in L1 sarcoma tumor tissue, not affecting tumor mass and tumor cells angiogenic activity.

VEGF is one of the most important factors responsible for stimulating angiogenesis during tumor growth. Its production and function may be suppressed by various polyphenolic compounds [14, 15], among them by phenolic



**Fig. 5.** Typical picture of tumor from the control group of mice. Numerous blood vessels at the tumor margin. Photo taken under 20 × lens



**Fig. 6.** Small blood vessels on the tumor margin and within a tumor (arrows). Typical picture for tumors collected from *Echinacea*-fed mice. Photo taken under 20 × lens

acids [16]. It is noteworthy, that *Echinacea purpurea* extracts contain cichoric, caftaric, chlorogenic and caffeic acids.

7-day old tumors were small and probably we observed the influence of *Echinacea* on the very early events of their growth - inhibition of VEGF production and lesser number of blood vessels at the tumor margin in *Echinacea*-fed groups of mice.

Previously we observed angioinhibitory effect of complex herbal remedy (syrup Alchinal), containing *Echinacea purpurea*, *Allium sativum* (garlic) and cocoa, as well as its inhibitory effect on sarcoma L-1 growth. However, in that study mice were fed drug for longer time (since -4 to +13 day, in respect to the day of tumor cells grafting). Moreover, the final effect was probably the resultant of action of all remedy compounds, as anti-angiogenic and anti-tumor activities of garlic and cocoa has been described [17].

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